

RECOMBINANT CELL LINEIntroduction

1 The invention also relates to recombinant cell lines
2 transformed to express a dimeric form of
3 interleukin, or a subunit thereof, and expression
4 vectors used to transform the cell lines. The
5 invention also relates to a method of screening
6 candidate compounds for the ability to inhibit
7 assembly and secretion of dimeric forms of
8 interleukins, or subunits thereof.

9

10 Background Art

11

12 Cytokines are a unique family of growth factors.
13 Secreted primarily from leukocytes, cytokines
14 stimulate both the humoral and cellular immune
15 responses, as well as the activation of phagocytic
16 cells. Cytokines secreted from lymphocytes are
17 termed lymphokines, whereas those secreted by
18 monocytes or macrophages are termed monokines. Many
19 of the lymphokines are also known as interleukins

1 (IL's), since they are not only secreted by
2 leukocytes, but are also able to affect the cellular
3 responses of leukocytes. Specifically, interleukins
4 are growth factors targeted to cells of
5 hematopoietic origin. One of the interleukins, IL-
6 12, is a pro-inflammatory cytokine interleukin. This
7 cytokine is predominantly secreted either as a $\alpha\beta$
8 heterodimeric form or as a $\beta\beta$ homodimeric form. Both
9 dimer forms bind the IL-12-receptor on target cells
10 but differ in the spectrum of biological activities
11 induced. The $\alpha\beta$ form is crucial for generation of
12 cell-mediated immunity against parasites, viruses
13 and bacteria, but contributes also to destructive
14 effects in pathogenesis of autoimmune diseases, e.g.
15 MS, RA and inflammatory bowel disease. The $\beta\beta$ form
16 has been shown to be instrumental in virus-induced
17 inflammation, and in excessive epithelial airway
18 inflammation seen in asthma. Thus, both forms of IL-
19 12 are disease-promoting factors in a variety of
20 conditions. Recently, two novel cytokines have been
21 discovered, named interleukin-23 and interleukin-27
22 that apparently belong to the IL-12 subclass of
23 cytokines based on structural relationships. Both
24 IL-23 and IL-27 share with IL-12 a typical
25 heterodimeric structure and are involved in a
26 similar array of immune responses.

27

28 Celebrex is a diaryl-substituted pyrazole. It is a
29 nonsteroidal anti-inflammatory drug (NSAID) that is
30 indicated for the treatment of osteoarthritis,
31 rheumatoid arthritis, for the management of acute
32 pain in adults for the treatment of primary

1 dysmenorrhea. The mechanism of action of CELEBREX is
2 believed to be due to inhibition of prostaglandin
3 synthesis, primarily via inhibition of
4 cyclooxygenase-2 (COX-2). Scientific literature
5 indicates that CELEBREX displays antitumor effects
6 by sensitizing cancer cells to apoptosis. A recent
7 paper has indicated that CELEBREX blocks the
8 endoplasmic reticulum (ER) Ca^{2+} -ATPases, and it has
9 been suggested that this Ca^{2+} perturbation may be
10 part of the signaling mechanism by which CELEBREX
11 triggers apoptosis. This Ca^{2+} perturbation effect
12 seems to be unique to CELEBREX and was not seen with
13 any of the other COX inhibitors (e.g. aspirin,
14 ibuprofen, naproxen etc.)

15

16 Statement of Invention

17

18 According to the invention, there is provided an
19 expression vector comprising DNA encoding a subunit
20 of a dimeric form of interleukin under
21 transcriptional control of an ecdysone-inducible
22 promoter.

23

24 Suitably, the subunit of a dimeric form of
25 interleukin is selected from the group comprising:
26 p35 (alpha) subunit of interleukin 12 (IL-12); p40
27 (beta) subunit of IL-12; p19 chain of IL-23; p40
28 subunit of IL-23; ebi3 subunit of IL-27; and p28
29 subunit of IL-27.

30

31 Typically, the vector comprises an ecdysone-
32 inducible mammalian expression plasmid, wherein the

1 DNA encoding the subunit of a dimeric form of
2 interleukin is included in the plasmid.

3

4 In one embodiment of the invention, the vector
5 comprises DNA encoding a p40 subunit of IL-12. Cell
6 lines stably transfected with such a vector will,
7 when induced, express both homodimeric IL-12 and the
8 beta-subunit of IL-12.

9

10 In another embodiment of the invention, the vector
11 comprises DNA encoding a p35 subunit of IL-12. Cell
12 lines stably transfected with such a vector will,
13 when induced, express the alpha-subunit of IL-12.

14

15 In another embodiment of the invention, the vector
16 comprises DNA encoding a p19 subunit of IL-23. Cell
17 lines stably transfected with such a vector will,
18 when induced, express the p19 subunit of IL-23.

19

20 In a preferred embodiment of the invention, the
21 ecdysone inducible mammalian expression vector is
22 selected from the group comprising: pIND; pIND(SP1);
23 and pINDHygro.

24

25 In a particularly preferred embodiment of the
26 invention, the DNA encoding a subunit of dimeric
27 interleukin 12 includes a DNA sequence encoding a 6
28 x histidine tag.

29

30 In one embodiment of the invention, the expression
31 vector is selected from the group comprising: pIND-

1 p35H; pIND(SP1)-p35H; pIND-40H; pINDHygro-p40;
2 pIND(SP1)-p40H; and pIND-p40.

3

4 Suitably, the DNA encoding the subunit of dimeric
5 interleukin is digested with *NheI* and *XhoI*
6 restriction enzymes prior to ligation of the
7 digested DNA products into the expression vector.

8

9 The invention also relates to an expression vector
10 pIND(SP1)-p35H having ECACC accession number
11 03120401. A sample of this vector was deposited at
12 the ECACC on 4 December 2003.

13

14 The invention also relates to a method a producing a
15 tightly controlled expression vector capable of
16 transforming a host cell which when transformed is
17 capable of producing a recombinant dimeric
18 interleukin, or a subunit thereof, under
19 transcriptional control of a ecdosone inducible
20 promoter, comprising the steps of:

- 21 - providing cDNA for a subunits of a dimeric
- 22 interleukin;
- 23 - digesting the cDNA with at least one
- 24 restriction enzyme; and
- 25 - ligating the digested cDNA product into an
- 26 ecdysone-inducible mammalian expression vector.

27

28 In a preferred embodiment of the invention, the DNA
29 is digested with two restriction enzymes, these
30 being *NheI* and *XhoI*. Suitably, the plasmid into
31 which the digested DNA is to be ligated is also
32 digested with the same restriction enzymes.

1

2 The invention also relates to an expression vector
3 obtainable by the method of the invention.

4

5 The invention also relates to a cell line
6 transfected with at least one expression vector of
7 the invention, wherein the DNA encoding the at least
8 one subunit of a dimeric interleukin is under
9 transcriptional control of a ecdysone-inducible
10 mammalian expression system.

11

12 Suitably, the ecdysone-inducible mammalian
13 expression system comprises a plasmid other the
14 expression vector of the invention which
15 constitutively expresses two receptors which
16 interact in the presence of ecdysone, or an analog
17 thereof, to form a complex which binds to a response
18 element of a promotor controlling DNA encoding the
19 at least one subunit of a dimeric interleukin. Such
20 a plasmid is sold by Invitrogen under the name
21 pVgRxR.

22

23 In one embodiment, the cell line is transfected with
24 DNA that encodes a p35 (beta) subunit of IL-12. Such
25 a cell line, when induced, produces homodimeric IL-
26 12 and the beta-subunit of IL-12.

27

28 In another embodiment, the cell line is transfected
29 with an expression vector which includes DNA
30 encoding the p40 subunit of IL-12, and a further
31 expression vector which includes DNA encoding the

1 p35 subunit of IL-12. Such a cell line, when
2 induced, produces heterodimeric IL-12.

3

4 In another embodiment, the cell line is transfected
5 with an expression vector which includes DNA
6 encoding the p40 subunit of IL-12 (which is
7 identical to the p40 subunit of IL-23), and a
8 further expression vector which includes DNA
9 encoding the p19 subunit of IL-23. Such a cell line,
10 when induced, produces heterodimeric IL-23.

11

12 Typically, the cell lines of the invention include
13 the plasmid pVgRxR.

14

15 In one embodiment of the invention, the cells of the
16 cell line are human embryonic kidney cells,
17 preferably EcR293 cells.

18

19 The invention also relates to a cell line according
20 to the invention in which the cells are natural
21 beta-subunit-producing cells such as a HIBERNIA1
22 cell line.

23

24 The invention also relates to a cell line having
25 ECACC accession number 03112701. This cell line
26 includes an expression vector having DNA encoding
27 for the p40 (beta) subunit of IL-12. A deposit of
28 the recombinant cells was made at the ECACC on 27
29 November 2003.

30

31 The invention also relates to a method of producing
32 a cell line capable of producing a recombinant

1 dimeric interleukin, or a subunit thereof, under
2 transcriptional control of a ecdysone-inducible
3 promoter, comprising the steps of:

- 4 - providing at least one expression vector
5 according to the invention; and
- 6 - transfecting a host cell with the at least one
7 expression vector,
8 wherein the DNA encoding the at least one subunit
9 of a dimeric interleukin is under the
10 transcriptional control of a ecdysone-inducible
11 mammalian expression system.

12

13 The invention also relates to a method of preparing
14 cDNA encoding a subunit of a dimeric form of
15 interleukin comprising the steps of providing cDNA
16 encoding the subunit, and digesting the cDNA with
17 restriction enzymes *NheI* and *XhoI* to obtain a cDNA
18 product.

19

20 The invention also relates to a method of screening
21 a candidate compound for the ability to inhibit
22 dimer assembly and secretion of a dimeric form of
23 interleukin, comprising the steps of:

- 24 - incubating a cell culture comprising a cell
25 line of the invention with the candidate
26 compound;
- 27 - inducing transcription of the dimeric
28 interleukin in the cells of the culture using
29 ecdysone or an ecdysone analog; and
- 30 - assaying the cell culture for the presence of
31 secreted interleukin.

32

1 In one embodiment of the method, the interleukin
2 expressed by the cell line has a 6 x histidine amino
3 acid sequence tagged on either or both of the
4 subunits thereof, wherein the assaying step involves
5 Ni-NTA affinity chromatography.

6 Alternatively, the assaying step involves probing
7 the cell culture with an antibody specific to a
8 dimeric form of interleukin, or a subunit thereof.

9

10 The invention also relates to an inhibitor of dimer
11 assembly and secretion of dimeric interleukin
12 identified by the method of the invention.

13

14 The invention also relates to a method of prevention
15 or treatment of inflammatory disease comprising a
16 step of treating an individual with an inhibitor
17 identified by the method of the invention. One such
18 inhibitor IDENTIFIED is CELEBREX.

19

20 In a further aspect, the invention provides a method
21 of treating disease having a pathogenesis which
22 includes endogenous production of any of cytokines
23 IL-12, IL 23 or IL-27, the method comprising a step
24 of treating an individual with an endoplasmic
25 reticulum (ER) Ca^{2+} perturbation reagent.

26

27 In a further aspect, the invention provides the use
28 of an ER Ca^{2+} perturbation reagent in the manufacture
29 of a medicament for the treatment of disease having
30 a pathogenesis which includes endogenous production
31 of any of cytokines IL-12, IL-23 or IL-27.

32

1 In a further aspect, the invention provides the use
2 of an ER Ca^{2+} perturbation reagent for the treatment
3 of disease having a pathogenesis which includes
4 endogenous production of any of cytokines IL-12, IL-
5 23 or IL-27.

6

7 In a further aspect, the invention relates to a
8 method of inhibiting the formation of one or more
9 cytokines in an individual, which method comprises
10 the step of treating an individual with ER Ca^{2+}
11 perturbation reagent. In one embodiment, the
12 cytokines are selected from IL-12, IL-23 and IL-27.

13

14 In a further aspect, the invention relates to the
15 use of an ER Ca^{2+} perturbation reagent to inhibit the
16 formation of one or more cytokines in an individual.
17 In one embodiment the cytokines are selected from
18 IL-12, IL-23 and IL-27.

19

20 In a preferred embodiment, the disease is an
21 inflammatory disease. More preferably, the disease
22 is a disease in which one or more endogenously
23 produced IL-12 forms play a disease promoting role.
24 Typically, the IL-12 forms are $\alpha\beta$ heterodimeric and
25 $\beta\beta$ homodimeric forms.

26

27 In one embodiment, diseases in which cyclooxygenase-
28 2 (COX-2) is reported to play a substantial disease
29 promoting role are disclaimed.

30

31 In one embodiment, the inflammatory disease is a
32 disease in which the endogenous production of one or

1 both of $\alpha\beta$ and $\beta\beta$ forms of IL-12 is known to lead to
2 disease in a COX-2 independent manner.

3

4 The invention also relates to a method of inhibiting
5 the production of one or more cytokines in an
6 individual in a post-translational manner, which
7 method comprises a step of treating an individual
8 with ER Ca^{2+} perturbation reagent.

9

10 Preferably, the disease is selected from the group
11 consisting of infectious diseases; bacterial
12 protozoal or virus-induced inflammation; epithelial
13 airway inflammation such as asthma; allergic
14 disease; autoimmune disease such as MS, RA and
15 Inflammatory Bowel Disease; and -all conditions in
16 which endogenously produced IL-12 α/β or $\beta\beta$ forms
17 are thought to play a disease-promoting role,
18 including:

19

20 Pulmonary fibrosis

21 Pulmonary tuberculosis

22 Asthma

23 Sarcoidosis

24 Leprosy

25 Schistosomiasis

26 Lupus erythematosus

27 Lupus nephritis

28 Allograft rejection

29 Airway inflammation

30 Respiratory syncytial virus infection

31 Multiple sclerosis

32 Alzheimer's disease

- 1 Abortion (women with recurrent pregnancy loss)
- 2 Certain vaccines aimed at inducing TH2-type immune
- 3 responses
- 4 Experimental autoimmune myocarditis
- 5 Tuberculosis
- 6 Psoriatic arthritis
- 7 Rheumatoid arthritis
- 8 Osteoarthritis
- 9 Colonic inflammation (colitis)
- 10 Crohn's Disease
- 11 Inflammatory bowel disease
- 12 Atopic dermatitis, AD (chronic stage)
- 13 Inflammatory skin disease
- 14 Insulin dependent diabetes mellitus Type I and II
- 15 Endotoxaemia
- 16 Exposure to organic dust
- 17 Periodontal diseases
- 18 Nephrotic syndrome
- 19 Hepatocellular damage in chronic hepatitis C
- 20 Primary biliary cirrhosis
- 21 Cancer patients (Various cancers, and various stages
- 22 in cancer that are typically accompanied with
- 23 dysregulated IL-12, IL-23 and/or or IL-27
- 24 production)
- 25 ANCA associated vasculitis and sepsis
- 26 Experimental crescentic glomerulonephritis
- 27 Atherosclerosis
- 28 Acute viral myocarditis
- 29 Autoimmune myocarditis
- 30 Experimental autoimmune myasthenia gravis
- 31 Uveitis (as Behret's disease)
- 32 Thyroiditis and Grave's disease

- 1 Thyroid autoimmune disease
- 2 Myelopathy (HTLV-I-associated myelopathy)
- 3 Symptomatic transient hypogammaglobulinaemia of
- 4 infancy (THI)
- 5 Selective IgA deficiency (SIgAD)
- 6 Schizophrenia
- 7 Primary malignant melanoma
- 8 Abdominal aortic aneurysm
- 9 Autoimmune thrombocytopenic purpura
- 10 Heatstroke
- 11 Meningococcal sepsis
- 12 Septic shock
- 13 Meningoencephalitis
- 14 Bacterial meningitis
- 15 Pregnancy
- 16 Pre-eclampsia
- 17 HELLP syndrome (hemolysis, elevated liver function
- 18 test and low platelet counts
- 19 Endometriosis
- 20 Acute pancreatitis
- 21 Lung fibrosis induced by silica particles
- 22 Scleroderma
- 23 Sjogren's syndrome
- 24 Ankylosis spondylitis
- 25 Hashimoto's thyroiditis
- 26 Autoimmune anemias
- 27 Goodpasture's syndrome
- 28 Addison's disease
- 29 Autoimmune hemolytic anemia
- 30 Spontaneous infertility (sperm)
- 31 Poststreptococcal glomerulonephritis
- 32 Autoimmune neuritis (Guillain-Barre syndrome)

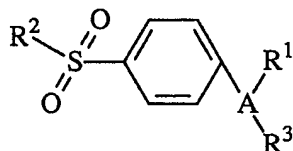
1 Sialadenitis
2 Brucellosis
3 Chickenpox and related viral diseases
4 Helicobacter Pyloris-induced gastritis
5 Common Variable Immunodeficiency (CVI)

6
7 In one embodiment, the disease is a conditions
8 characterized by dysregulation of IL-12, IL-23 or
9 IL-27 production conferred by polymorphisms in their
10 respective genes, or by polymorphisms in genes
11 involved in the biological activation or signal
12 transduction pathway of these cytokines.

13
14 In one embodiment, the ER Ca^{2+} perturbation reagent
15 is selected from the compounds of Formula I:

16

17 Formula I



18

19 wherein A is a substituent selected from partially
20 unsaturated or unsaturated hetrocyclyl and partially
21 unsaturated or unsaturated carbocyclic rings;
22 wherein R¹ is at least one substituent selected from
23 heterocyclyl, cycloalkyl, cycloalkenyl and aryl,
24 wherein R¹ is optionally substituted at a
25 substitutable position with one or more radicals
26 selected from alkyl, haloalkyl, cyano, carboxyl,
27 alkoxycarbonyl, hydroxyl, hydroxyalkyl, amino,
28 alkylamino, arylamino, nitro, alkoxyalkyl,
29 alkylsulfinyl, halo, alkoxy and alkylthio;

1 wherein R² is methyl or amino; and
2 wherein R³ is a radical selected from hydrido, halo,
3 alkyl, alkenyl, oxo, cyano, carboxyl, cyanoalkyl,
4 heterocyclyloxy, alkyloxy, alkylthio, alkylcarbonyl,
5 cycloalkyl, aryl, haloalkyl, heterocyclyl,
6 cycloalkenyl, aralkyl, hetrocyclylalkyl, acyl,
7 alkythioalkyl, hydroxyalkyl, alkoxycarbonyl,
8 arylcarbonyl, aralkylcarbonyl, aralkenyl,
9 alkoxyalkyl, arylthioalky, aryloxyalkyl,
10 aralkylthioalky, aralkoxyalkyl, alkoxyaralkoxyalkyl,
11 alkoxycarbonalkyl, aminocarbonyl,
12 aminocarbonylalkyl, alkyaminocarbonyl, N-
13 arylaminocarbonyl, N-alkyl-N-arylaminocarbonyl,
14 alkylaminocarbonylalkyl, carboxyalkyl, alkylamino,
15 N-arylamino, N-aralkylamino, N-alkyl-N-aralkylamino,
16 N-alkyl-N-arylamino, aminoalkly, alkylaminoalkyl, N-
17 arylaminoalkyl, N-aralkylaminoalkyl, N-alkyl-N-
18 aralkylaminoalky, N-alkyl-N-arylaminoalkyl, aryloxy,
19 aralkoxy, arylthio, aralkylthio, alkylsulfinyl,
20 alkylsulfonyl, aminosulfonyl, alkylaminosulfonyl, N-
21 arylaminosulfonyl, arylsulfonyl, N-alkyl-N-
22 arylaminosulfonyl; or a pharmaceutically-acceptable
23 salt thereof.

24

25 In a preferred embodiment, the ER Ca²⁺ perturbation
26 reagent is selected from the compounds and
27 compositions described in US Patent 5,972,986,
28 Column 3, line 34 to Column 10, line 32. In a
29 particularly preferred embodiment, the ER Ca²⁺
30 perturbation reagent is a diaryl- substituted
31 pyrazole marketed under the brand name CELEBREX
32 (Celecoxib). CELEBREX is chemically designated as 4-

1 [5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide.

3

4 Alternatively, the ER Ca^{2+} perturbation reagent may
5 be thapsigargin or A23187.

6

7 The invention will be more clearly understood from
8 the following description of some embodiments
9 thereof, given by way of example only, with
10 reference to the accompanying figures.

11

12 Brief Description of the Figures

13

14 Figure 1. is a schematic representation of the
15 Ecdysone-Inducible Mammalian Expression System.

16

17 Figure 2. is a schematic overview of the pIND,
18 pINDSP1 and pINDHygro vectors.

19

20 Figure 3. Primers used for amplification of the α
21 and β chains of IL-12. (A) α chain forward primer;
22 (B) α -chain reverse primer; (C) β chain forward
23 primer; (D) β -chain reverse primer and (E) β -chain
24 reverse primer without histidine tag. The sequence
25 coding for the hexahistidine-tag is represented in
26 red, while initiation and stop codons are indicated
27 in bold. The Kozak translation initiation sequence
28 is underlined.

29

30 Figure 4. Analysis of the amplification of the β -
31 chain from LPS-induced U937 cells by means of 1.5%

1 agarose gel electrophoresis. Lane 1, 100-bp DNA
2 marker; Lane 2-4, β -chain fragment amplified in the
3 presence of 2 mM MgSO_4 (lane 2); 3 mM MgSO_4 (lane 3)
4 or 4 mM MgSO_4 (lane 4).

5

6 Figure 5. Amplification of α -chain cDNA (702bp).
7 Lane 1, 100-bp DNA marker; Lane 2-4, α -chain
8 fragment amplified in the presence of Pwo DNA
9 polymerase and 2 mM MgSO_4 (lane 2); 3 mM MgSO_4 (lane
10 3) or 4 mM MgSO_4 (lane 4).

11

12 Figure 6. Amplification of β -chain cDNA (1029bp).
13 Lane 1, 100-bp DNA marker; Lane 2-4, β -chain
14 fragment amplified in the presence Pwo DNA
15 polymerase of 2 mM MgSO_4 (lane 2); 3 mM MgSO_4 (lane
16 3) or 4 mM MgSO_4 (lane 4). Lanes 1-3 correspond to
17 products obtained using the reverse primer without
18 the histidine tag and lanes 5-6 including the
19 histidine tag.

20

21 Figure 7. Expression cassettes for the α and β -
22 chains of IL-12 in the series of pIND vectors. (A)
23 Expression cassette shared by all vectors of the
24 pIND series with indication of the location of the
25 minimal heat shock promoter ($P_{\Delta\text{HSP}}$) and the bovine
26 growth hormone poly-adenylation signal (BGH pA); (B)
27 and (C) 5' and 3' nucleotide sequences and
28 corresponding amino- and carboxy-terminal amino acid
29 sequences of the recombinant α (B) and β (C) chains
30 with indication of the primer sequences.

31

1 Figure 8. Electrophoresis of amplification products
2 obtained by colony PCR of ampicillin-resistant
3 clones. The photographs show the results obtained
4 from clones transformed with (A) pIND(SP1)-p40H; (B)
5 pINDHygro-p40; and (C) pIND-p40.

6
7 Figure 9. Electrophoresis of amplification products
8 obtained by colony PCR of ampicillin-resistant
9 clones following transformation with pIND(SP1)-p35H

10
11 Figure 10. Confirmation of the presence of inserts
12 by means of restriction analysis of minipreps. (M)
13 100-bp ladder; (A) pIND(SP1)-p35H digested with *NheI*
14 and *XhoI* (insert of 700 bp); (B) pINDHygro-p40
15 digested with *NheI* and *XhoI*; and (C) pIND(SP1)-p40H
16 digested with *NheI* and *XhoI* (inserts of 900 bp).
17 Note: the vector portions were too large to
18 penetrate into this high-percentage agarose gel and
19 are therefore not visible.

20
21 Figure 11. Analysis of ponasterone A-inducible
22 expression of IL-12 α (A) and β (B) chains in
23 transfected cell lines. 4-15% reducing SDS-PAGE
24 analysis of clones 1A9 (His-tagged α -chain), 2G10
25 (His-tagged α -chain) and 3D9 (β -chain). (A)
26 detection with monoclonal anti-p35 antibody. 1 (lane
27 1), 5 (lane 2) and 10 (lane 3) μ l of the medium, and
28 1 (lane 4), 5 (lane 5) and 10 μ l (lane 6) of the
29 soluble cell lysate of ponasterone A-induced clone
30 1A9 were submitted to 4-15% SDS-PAGE and
31 immunoblotted. Lanes 7-12 represent similar
32 fractions of clone 2G10. (B) detection with

1 monoclonal anti-p40 antibody. Lanes 1-6: fractions
2 of medium and cell lysate of clone 3D9 as described
3 for (A); Lanes 7-12: cell lysates of clones 1A9 and
4 2G10, used as negative control.

5

6 Figure 12. Expression levels of the IL-12 α chain in
7 18 different neomycin-resistant EcR293 clones.
8 Anti- α -chain immunoblots of soluble cell lysates
9 were prepared from induced (I) and uninduced (U)
10 EcR293 clones obtained following transfection and
11 neomycin selection with (A) pIND-p35H; (B, C)
12 pIND(SP1)-p35H and (D) pIND-p35H or pIND(SP1)-p35H.
13 Lysates were subjected to reducing SDS-PAGE using 4-
14 15% gels, blotted and immunodetected with anti α -
15 chain antibody. As negative control, we used the
16 secreted fraction of clone 4B6Z, which expresses the
17 β -chain (lane 13-14 in Figure 16D).

18

19 Figure 13. Expression levels of the IL-12 β chain in
20 hygromycin- (A) and neomycin- (B) resistant EcR293
21 clones. Anti- β -chain immunoblots of soluble cell
22 lysates prepared from induced (I) and uninduced (U)
23 EcR293 cells. Clones were obtained by transfection
24 with (A) pINDHygro-p40; or (B) pIND(SP1)-p40H.
25 Lysates were subjected to SDS-PAGE using 4-15% gels,
26 blotted and immunodetected with anti α -chain
27 antibody.

28

29 Figure 14. Transient transfection of HIBERNIA.1
30 cells with pIND(SP1)-p35H. Non-reducing 4-15% SDS-
31 PAGE and immunoblot of secreted fractions of the

1 transfected cell line following 30 (lanes 1 and 2)
2 and 48 (lanes 3 and 4) hrs of induction with
3 ponasterone A. The cells were transfected with 1
4 (lanes 1 and 3) or 2 (lanes 2 and 4) μ g of
5 pIND(SP1)-p35H. As a control the secreted fraction
6 of the non-transfected induced β -chain-producing
7 HIBERNIA.1 cells was used (lane 5). (A) detection
8 with anti β -chain antibody; (B), detection with anti
9 α -chain antibody.

10

11 Figure 15. Immunodetection of α and β subunits of
12 IL-12 in medium of HIBERNIA.1 cells transiently
13 transfected with pIND(SP1)-p35H following reducing
14 SDS-PAGE. Lane 1, detection with anti α -chain
15 antibody; Lane 2, detection with anti- β -chain
16 antibody, Lane 3, detection with both antibodies at
17 the same time.

18

19 Detailed Description of the Invention

20

21 Recombinant cell lines that secrete various forms of
22 IL-12 under control of tightly regulated promoters
23 were generated. It was observed that treatment of
24 these cell lines with an ER Ca^{2+} perturbation reagent
25 such as thapsigargin inhibited secretion of both the
26 $\alpha\beta$ and $\beta\beta$ forms of IL-12. The compound CELEBREX was
27 also tested on assembly of IL-12, and found that it
28 exerts a similar inhibitory effect on the secretion
29 of the $\alpha\beta$ and $\beta\beta$ forms of IL-12. There is a total
30 block in the secretory production of both dimer
31 forms of IL-12, and maximal effects are obtained

1 with the normal physiological working concentration
2 of CELEBREX in the absence of any apparent toxic
3 effects as measured with the MTT assay. These
4 affects are conferred in a post-transcriptional and
5 post-translation manner as there is no effect on
6 mRNA of IL-12. Without being bound by theory,
7 evidence has been produced to support a Ca^{2+} -
8 dependent disturbance in the folding pathway of IL-
9 12 due to impaired activity of certain chaperones in
10 the ER.

11

12 The inhibitory effect of CELEBREX on formation of
13 the $\alpha\beta$ and $\beta\beta$ forms of IL-12 in vitro indicates that
14 this drug is of interest for the treatment of
15 inflammatory conditions in which endogenous
16 production of these IL-12 forms is known to lead to
17 disease in a COX2-independent manner, including MS,
18 IBD, virus-induced inflammation and asthma.

19

20 IL-12 is a member of a family of cytokines that
21 includes two recently discovered members IL-23 and
22 IL-27. All of these cytokines have a typical
23 heterodimeric structure and display an array of both
24 overlapping and distinct activities. It is thought
25 that also IL-23 and IL-27 may contribute to
26 destructive inflammation in various conditions.
27 Most anti-cytokine drugs work by inhibiting
28 transcription of mRNA. To our knowledge this is the
29 first demonstration of a drug that inhibits cytokine
30 formation in a post-translational manner on the
31 level of folding and secretion of the protein, i.e.
32 by perturbation.

1

2 Experimental methods

3

4 Materials. Celecoxib (Celebrex) was obtained from
5 Hefei Sceneri Chemical Co.; thapsigargin was
6 obtained from Calbiochem and A23187 from Sigma.

7

8 Cell culture. HEK293 IL-12 β/β and α/β producing
9 cell lines were maintained in a CO₂ incubator at 37
10 °C (5% CO₂). Cells were cultured in DMEM medium
11 supplemented with 10% foetal bovine serum.

12

13 Cloning and expression of the α and β chain of IL-12

14

15 Extraction of mRNA from IL-12 producer cell line

16

17 Human monocytic U937 cells were kindly provided by
18 the Rega Institute, Leuven, Belgium. U937 cells were
19 grown in DMEM (Dulbecco's modified eagle medium)
20 supplemented with 10% FBS, 2 mM L-glutamine
21 (LifeTechnologies) and 50 μ g/ml of gentamycin
22 (Sigma). Cells were cultivated in 75cm² flasks, in a
23 CO₂ incubator (5% CO₂) at 37°C and subcultured once a
24 week by splitting 1/10 by means of trypsination with
25 Trypsin-EDTA (LifeTechnologies) followed by
26 centrifugation to remove trypsin. Cells were induced
27 with IFN- γ (100 ng/ml) and LPS (1 μ g/ml; Sigma) for
28 24 hours. Total RNA was extracted from cells (10⁷)
29 using StrataPrp[®] Total RNA Miniprep kit
30 (Stratagene). This method uses a powerful
31 denaturant, guanidine thiocyanate, in the lysis
32 buffer. Afterwards, the sample was filtrated to

1 reduce the amount of DNA and subjected to a silica-
2 based fibre matrix to capture RNA.

3

4 Amplification of α and β -chains of IL-12 by RT-PCR

5

6 To perform RT-PCR on the RNA extracted from IL-12
7 producer cells, we used the ProSTAR™ HF Single-Tube
8 RT-PCR System (High Fidelity) obtained from
9 Stratagene. This method uses the StrataScript
10 reverse transcriptase, which is subsequently
11 inhibited by incubation at 95°C. Amplification is
12 achieved with TaqPlus Precision polymerase.
13 Oligonucleotides complementary to the sequences to
14 be amplified (α and β -chain) were synthesized by
15 LifeTechnologies. For the α -chain, the forward
16 primer was designed to contain the second initiation
17 methionine (ATG) and NheI restriction site (GCTAGC),
18 while the reverse primer contained the stop codon
19 (TAA), XhoI restriction site (CTCGAG) and a 6x
20 Histidine tag sequence [3x(ATGGTG)]. The β -chain
21 forward primer contained the initiation codon and
22 the NheI restriction site as well. We synthesized
23 two different oligonucleotides as reverse primers.
24 The first one contains the stop codon, XhoI
25 restriction site and the 6xHis sequence, and the
26 second was designed without the 6xHistidine
27 sequence.

28

29 α -chain

30

31 Forward 5' CAGGCTAGCGCAGCCATGTGTCCAGCGCGCAGC3'

32 Reverse 5' CTGCTCGAGTTAATGGTGATGGTGATGGTGGAAGCA

1 TTCAGATAGCT3'

2 β-chain

3

4 Forward 5'CAGGCTAGCGCAGCCATGTGTTTACCAGCAGTTG3'

5 Reverse 5'CTGCTCGAGCTAATGGTGATGGTGATGGTGACTGCAG

6 GGCACAGATG3'

7 Reverse 5'CTGCTCGAGCTAACTGCAGGGGCACAGATG3

8

9 The DNA sequences of the above primers are provided
10 as Sequence ID No's 1 to 5 in the Sequence Listing
11 Section of this specification.

12

13 The RT-PCR reaction mix contained 5 µl of 10×HF RT-
14 PCR buffer, 100 ng of forward primer; 100 ng of
15 reverse primer, 200 µM of dNTP, 100 ng of RNA, 1 U
16 of StrataScript RT (1 unit), and the Tagplus
17 Precision DNA polymerase

18

19 RT-PCR conditions were:

20	42°C	30 min	1 cycle
21	95°C	1 min	1 cycle
22	95°C	30 sec	30 cycles
23	55°C	30 sec	
24	68°C	2 min	
25	68°C	10 min	1 cycle
26	4°C	∞	

27

28 The RT-PCR products were analyzed by means of 1.5%
29 agarose gel electrophoresis coupled to staining in
30 ethidium bromide for 30 minutes. The products were
31 visualized on an UV transilluminator.

32

1 Amplification of the α and β -chains of IL-12
2 starting from the cDNAs

3
4 The cDNAs coding for the β -chain (p40) and α -chain
5 (p35) of interleukin-12 were obtained from ATTC
6 (American Type Tissue Culture Collection, N 40854)
7 and HGMP Resource Centre (Human genome mapping
8 project, Image Clone 1932948, www.hgmp.mrc.ac.uk),
9 respectively. Pwo DNA polymerase from Boehringer
10 Mannheim was the enzyme used for amplification. This
11 enzyme has 3'-5' exonuclease proofreading activity.
12 Amplification was performed for 20 cycles (1 min at
13 95°C, 1 min at 47°C and 1 min at 72°C), using
14 different concentrations of MgSO_4 (2, 3 and 4 mM),
15 200 μM dNTP (Pharmacia), 600 nM of each primer and
16 50 ng of template DNA. A Bio-Rad thermocycler was
17 used for amplification of these products, and the
18 primers used were the same as indicated above.

19

20 Purification of PCR products

21

22 PCR products were purified by means of
23 phenol/chloroform extraction. An identical volume of
24 phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v)
25 was added to the samples. Samples were vortexed for
26 1 min and centrifuged at 18,000 rpm for 3 min, in
27 order to separate the different phases.
28 Subsequently, the aqueous phase was collected
29 carefully. We removed the primers with cleaning
30 columns from QIAGEN. As an alternative to the use of
31 QIAGEN columns, ethanol precipitation was performed
32 by adding 3 volumes of ethanol to the samples. 1/10

1 volume of sodium acetate (pH=5) was added to the
 2 reactions. Samples were left at -20° C for 1 hour,
 3 and a DNA pellet was obtained by centrifugation at
 4 18,000 rpm for 10 min at 4° C. Pellets were washed
 5 two times with 1 ml of 70% ethanol to remove salt
 6 and any organic molecules. The pellet was dried at
 7 room temperature and resuspended in 15 µl of TE
 8 buffer.

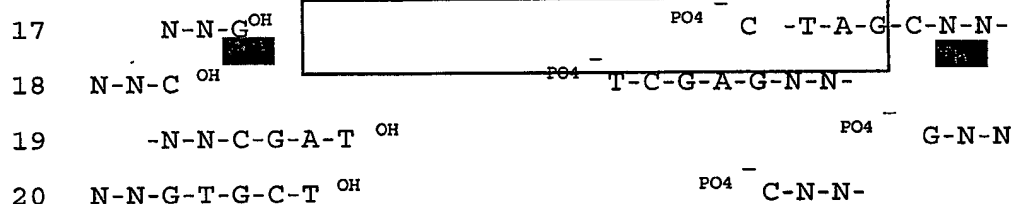
9

10 Restriction digestion of the α and β-chains

11

12 The PCR products were digested with the restriction
 13 enzymes NheI and XhoI which recognise the sequences
 14 G↓CTAGC and C↓TCGAG, respectively. Both restriction
 15 endonucleases were supplied by Amersham Pharmacia.

16



21

22

23 One µl of each enzyme (8 and 9 units respectively)
 24 and 2 µl of 10x OPA⁺ (One-Phor-All Buffer Plus)
 25 buffer were added to 16 µl of purified PCR product,
 26 to make up a final volume of 20 µl. The reactions
 27 were incubated at 37°C for 1.5 hours. The digestion
 28 was finalized by heat inactivation of the enzyme
 29 during 20 minutes at 65°C followed by incubation at
 30 room temperature for 20 min. To concentrate the
 31 digestion products by precipitation, 1/10 volume of

1 sodium acetate (pH=5) and ethanol were added to the
2 reactions. Samples were left at -20°C for 1 hour,
3 and the pellet was obtained by centrifugation at
4 18,000 rpm for 10 min at 4°C. The pellet was washed
5 2 times with 1 ml of 70% ethanol. The pellet was
6 allowed to dry at room temperature and resuspended
7 in 15 µl of TE buffer.

8
9 The purified PCR products were subjected to 1.5%
10 agarose gel electrophoresis in TBE buffer (45 mM
11 Tris-Borate, 1 mM EDTA) and the bands (700 bp for α-
12 chain and 900 bp for β-chain) were visualized after
13 staining in TBE buffer supplemented with 0.5 µg/ml
14 ethidium bromide (30 min) on a UV trans-illuminator.

15
16 Restriction digestion of pIND, pIND(SP1) and
17 pINDHygro vectors

18
19 The pIND, pIND(SP1) and pINDHygro vectors (ecdysome-
20 inducible mammalian expression vectors) were
21 supplied by Invitrogen. These vectors each contain
22 an ampicillin resistance gene for selection in E.
23 coli cells, and either a neomycin (only pIND and
24 pIND(SP1)) or an hygromycin resistance gene
25 (pINDHygro) for selection in mammalian cells. 2 µg
26 of each vector were digested with 8 units of NheI
27 and 9 units of XhoI, in 1x OPA buffer in a final
28 volume of 20 µl. Reactions were incubated at 37°C
29 for 1.5 hours and heat-inactivated at 65°C for 20
30 min. The vector DNA was precipitated as described
31 above.

32

1 Ligation of the α -chain into pIND and pINDSP1, and
2 of the β -chain into pINDSP1 and pINDHygro

3

4 Ligation of the digested PCR products (α and β -
5 chains) into digested vectors was catalyzed by T₄
6 DNA ligase enzyme (Promega). Two different ratios of
7 vector/insert (1:3 and 1:6) were tested in order to
8 optimize the ligation reaction. The reactions were
9 performed in a final volume of 20 μ l, containing 2
10 μ l of 10x T₄ ligase buffer, 1.5 units of T₄ DNA
11 ligase, 3 μ l of vector (100 ng), and the insert and
12 vector DNA. The reactions were incubated overnight
13 at 16°C.

14

15 Preparation of competent cells

16

17 E. coli JM109 (endA1, recA1, gyrA96, thi, hsdR17
18 (r_k⁻, m_k⁺), relA1, supE44, Δ (lac-proAB), [F',
19 traD36, proAB, lacI^q Δ M15] cells were made competent
20 by means of the CaCl₂ method (REF). A single clone
21 was inoculated in 5 ml of LB (Luria-Bertani broth
22 containing 10 g/l bactotryptone, 5 g/l bacto-yeast
23 extract and 10 g/l NaCl) medium and left overnight
24 with vigorously shaking at 37°C in a dedicated
25 incubator. An aliquot of this culture (100 μ l) was
26 added to 5 ml of LB (Luria B) medium. This culture
27 was further incubated at 37°C until an OD (A₆₀₀) of
28 0.5 was reached (log phase). Cells were placed on
29 ice for 5 minutes and then distributed (1 ml) in
30 sterilized eppendorf tubes. These tubes were
31 centrifuged at 13,000 rpm for 5 minutes,
32 supernatants were discarded and pellets were

1 resuspended in 1 ml of ice-cold CaCl_2 . The cells
2 were pelleted by centrifugation at 13,000 rpm for 5
3 minutes at 4°C, and washed in 1 ml of ice-cold
4 CaCl_2 ; the pellet obtained was now resuspended in
5 200 μl of CaCl_2 and frozen at -70°C.

6

7 Transformation of E. coli cells

8

9 Transformation was performed by mixing an aliquot of
10 competent cells with the ligation reactions (7.5
11 μl). This mixture was incubated on ice for 1 hour
12 and then subjected to a heat-shock at 42°C for 2
13 minutes. 1 ml of LB medium was added, and this
14 suspension was left at 37°C for 1 hour with
15 vigorously shaking. The transformation reactions
16 were mixed with 0.7 % agar supplemented with 50
17 $\mu\text{g}/\text{ml}$ ampicillin and then plated on preheated (37°C)
18 LB 1.5 % agar plates containing ampicillin (50
19 $\mu\text{g}/\text{ml}$). The plates were incubated overnight in an
20 incubator at 37°C.

21

22 Plamid purification from transformed E. coli cells

23

24 Colonies were inoculated in 5 ml of LB medium
25 containing 50 $\mu\text{g}/\text{ml}$ of ampicillin and left overnight
26 with vigorously shaking at 37°C in an incubator.
27 Cells were collected by centrifugation at 6,000 rpm
28 for 5 min. Pelleted cells were processed with the
29 Qiagen miniprep purification kit. Qiagen plamid
30 purification kits are based on an alkaline lysis
31 procedure using a buffer composed of SDS, that
32 disrupt the cell membranes, and NaOH, known to

1 denature genomic DNA. The cell lysate is loaded onto
2 an anion exchange resin that captures the DNA.
3 Afterwards, RNA, proteins, dye and impurities are
4 removed with a medium salt buffer (1 M NaCl). DNA is
5 eluted by means of a buffer that contains 1.25 M
6 NaCl. The eluted DNA is concentrated and
7 precipitated with isopropanol.

8

9 Sequencing of pIND(SP1)-p35H, pIND-p35H, pIND-40H,
10 pINDHygro-p40, pIND(SP1)-p40H and pIND-p40

11

12 The sequence of inserts was verified by the
13 enzymatic dideoxy-method described by Sanger et al.
14 (1977). The 'Ecdysone Forward' and 'BGH Reverse'
15 primers were used for forward and reverse
16 sequencing, respectively. The ABI PRISM Big DYE
17 Terminator Cycle Sequencing Ready Reaction Kit was
18 used. A mixture was prepared consisting of 8 µl of
19 the Terminator Ready Reaction Mix, 3.2 pmol of each
20 primer and 500 ng of DNA, and deionized water was
21 added to a volume of 20 µl. PCR conditions were 25
22 cycles 15 sec at 50°C, 25cycles

23 60°C for 4min

24 4°C ∞

25

26 Prior to sequencing, PCR products were purified in
27 order to remove dNTPs, primers and unincorporated
28 dye terminators. Ethanol precipitation was carried
29 out by adding 2 µl of 3 M sodium acetate pH=4.6, and
30 50 µl of 95 % ethanol to the PCR products. Samples
31 were vortexed and left at room temperature for 15
32 minutes. Subsequently, the samples were centrifuged

1 at 18,000 rpm (4°C) for 20 minutes. The supernatant
2 fractions were discarded and the pellet was washed
3 two times with 270 µl of 70 % ethanol. The pellet
4 was dried at room temperature, followed by
5 resuspension in 5 µl deionized formamide and 25 mM
6 EDTA to which blue dextran was added (50 mg/ml). The
7 samples were heated at 95°C for 2 minutes before
8 being loaded on an ABI PRISM 310 Genetic Analyzer.

9

10 Cell cultivation and transfection

11

12 Maintenance of cells

13

14 The human embryonic kidney cell line (EcR-293),
15 previously transfected with a pVgRXR construct that
16 encodes the regulatory ecdysone receptor, was
17 obtained from Invitrogen. The cells were cultured in
18 DMEM (LifeTechnologies) supplemented with 10 % of
19 foetal bovine serum (LifeTechnologies) and L-
20 glutamine 2 mM, in addition to 400 µg/ml zeocin, 400
21 µg/ml hygromycin or 600 µg/ml G418 for selection of
22 transfected cells (Invitrogen). Cells were
23 cultivated in 75 cm² flasks until 80% of confluency
24 was reached. Medium was removed and trypsin-EDTA
25 solution was added. After 15 minutes at 37 C, medium
26 was added and cells were collected. The suspensions
27 were centrifuged at 1,000 rpm for 5 min. in order to
28 remove the trypsin. Cells were resuspended in medium
29 and transferred to new culture flasks. Cells were
30 generally split 1 over 10 once a week. Cells were
31 maintained in a CO₂ incubator at 37°C (5% CO₂).

32

1 Freezing of EcR-293 clones expressing IL-12 α or
2 β -chains

3
4 Selected clones were cultivated in 175 cm²-flasks
5 until they reached 80 % confluency. The cells were
6 collected by trypsinization, and counted in a
7 hemacytometer by means of the trypan blue exclusion
8 assay - REF). Cells were resuspended at a density
9 of 3×10^6 cells/ml in the freezing medium, which was
10 composed of 90 % medium and 10% DMSO, and these
11 suspensions were transferred to cryovials. The
12 cryovials (LifeTechnologies) were placed at -20°C
13 for 2 hours, transferred to a -70°C freezer for 16
14 hours and, finally, placed in liquid nitrogen for
15 long-term storage.

16

17 Transfection of mammalian cells

18

19 Plasmid DNA used for transfection of mammalian cells
20 was purified by means of the Endofree kit of QIAGEN.
21 The purified plasmid DNA was quantified by
22 spectrophotometry. DNA concentrations were
23 determined by measuring absorbance at 260 nm, and
24 the purity was estimated by the A_{260}/A_{280} ratio.

25

26 EcR293 cells were plated in 6-well plates (2×10^5)
27 the day before the transfection. Transfections of
28 EcR293 cells were performed by means of the FuGENE-6
29 transfection reagent (Boehringer Mannheim). FuGENE-6
30 is a cationic lipid reagent which interacts with
31 negatively charged DNA to form a complex that can
32 cross the cell membrane. We used 1 or 2 μ g of

1 plasmid DNA (pIND(SP1)-p35H, pINDHygro-p40 or pIND-
2 p40H) to transfect cells. DNA samples were mixed
3 with 3 µl of FuGENE-6, and diluted in 97 µl of
4 medium. This solution was directly added to the
5 cells.

6

7 Preparation of soluble and insoluble fraction of
8 cells

9

10 Monolayers of EcR293 cells were washed 3 times with
11 large volumes of PBS. Cells were scraped and
12 resuspended in PBS, and centrifuged. The pelleted
13 cells were resuspended in lysis buffer, and
14 incubated on ice for 30 minutes. Lysis buffer was
15 composed of PBS, supplemented with 5 mM EDTA, 5 mM
16 EGTA, 1xprotease inhibitors (Boehringer Mannheim),
17 and 1% Triton X-100. Subsequently, the samples were
18 centrifuged at 18,000 rpm for 10 minutes, and the
19 soluble fraction recovered. The insoluble fraction
20 was washed with PBS supplemented with 1% Triton X-
21 100, and centrifuged at 18,000 rpm for 10 minutes.
22 Both the soluble and insoluble fractions were now
23 ready for analysis by SDS-PAGE and immunoblot.

24

25 Gel electrophoresis (SDS-PAGE)

26

27 Sodium dodecyl sulphate polyacrylamide
28 electrophoresis (SDS-PAGE; Laemmli, 1970) was used
29 as a standard technique for separating proteins in
30 the culture medium, soluble/insoluble cell
31 fractions, and immunoprecipitates. Generally,
32 protein samples were mixed with 2x SDS-PAGE loading

1 solution and loaded into the wells of pre-cast 4-15%
2 polyacrylamide gels. Electrophoresis was performed
3 at high voltage (200V) using a BioRad Mini-Protean
4 III electrophoresis unit and a Pharmacia power
5 supply. The electrophoresis buffer used contained 25
6 mM Tris, 192 mM glycine, and 0.1 % SDS (pH=8.3).
7 Size standards, such as the 'Perfect Protein Western
8 Blot Marker' from Novagen, were included in every
9 gel.

10

11 Western blotting, antibodies and detection

12

13 Immunoblot

14

15 Following SDS-PAGE, proteins were transferred from
16 the gel to a PVDF membrane by semi-dry
17 electroblotting. The polyacrylamide gel and 2 stacks
18 of pre-cut Whatman filter papers were equilibrated
19 in transfer buffer (48 mM Tris, 39 mM glycine, 0.04
20 % SDS, 20 % methanol) for 10 minutes. A PVDF
21 membrane was briefly soaked in methanol. The gel and
22 the PVDF membrane were placed between two stacks of
23 ten layers of filter papers, and the whole was
24 transferred to an electro-blotting unit. The
25 electrotransfer conditions applied were 0.8 mA/cm²
26 for 1 hour. The apparatus was dismantled, and the
27 membrane was incubated overnight at 4°C in blocking
28 buffer (2 % casein in TBS consisting of 10 mM Tris-
29 HCl, pH=7.4, and 100 mM NaCl). The membrane was
30 incubated with a primary antibody. We used the
31 following antibodies: (i) mouse α -p35 antibody G161-
32 566, obtained from BD-PharMingen, and used at a

1 working concentration amounting to 1/10,000 of the
2 original stock; (ii) mouse α -p40 antibody C8.6, BD-
3 PharMingen, used at a 1/5,000 dilution; or (iii) the
4 mouse anti-IL-12 antibody 1-2A1 obtained from Abcam,
5 1/1,000 diluted. For detection of chaperones we used
6 the following antibodies: (i) anti calreticulin, and
7 (ii) anti - GR894, from Stratogen.

8

9 These primary antibodies were added to TBS-T, i.e.
10 TBS supplemented with 0.5% Tween-20 and 0.1 %
11 casein. Incubation was done at room temperature for
12 2 hrs. Membranes were washed repeatedly with TBS-T
13 buffer (without casein), and subsequently incubated
14 with a secondary antibody. The secondary antibody
15 used was either goat anti-mouse or goat anti-rabbit
16 horseradish-peroxidase-conjugated antibody from
17 Jackson&ImmunoResearch (used at a 1/20,000
18 dilution). Incubation was performed for 1 hour at
19 room temperature, after which membranes were washed
20 again. The 'Perfect Protein Western Blot Marker' was
21 detected by means of an S-protein HRP conjugate
22 (Novagen), used at a working concentration of
23 1/5,000 of the original stock. Detection of poly-
24 histidine tagged fusion proteins was carried out
25 using the INDIATM HisProbe-HRP purchased from Pierce.
26 In this case, following overnight blocking, the
27 membrane was incubated with INDIA HisProbe (1/5,000
28 dilution) in TBS-T buffer with 0.1 % casein.

29

30 Chemiluminiscent detection

31

1 Chemiluminiscent detection was carried out with
2 either the 'ECL' or 'ECL+Plus' kit , both purchased
3 from Amersham-Pharmacia. The ECL detection principle
4 is based on the oxidation of luminol (cyclic
5 diacylhydracide), while ECL+Plus uses the enzymatic
6 generation of an acridinium ester. The latter
7 produces a more intense light emission of longer
8 duration. According to the manufacturer, the ECL kit
9 can generally detect 1 pg of antigen, while the
10 ECL+Plus kit can detect 20 times less protein. When
11 using the ECL kit, the working solution was prepared
12 by mixing equal parts of the 'Luminol/Enhancer' and
13 'Peroxidase' solutions. When using the ECL+Plus kit,
14 the working solution was prepared by mixing 40 parts
15 of the 'Substrate' solution with 1 part of 'Acridan'
16 solution. The membrane was incubated with these
17 solutions for 5 or 1 minute(s), respectively. Excess
18 solution was removed from the membrane. The membrane
19 was wrapped in cling film, and exposed using Kodak
20 MR1 or MR2 films.

21

22 Stripping and reprobing of membranes

23

24 Primary and secondary antibodies were removed from
25 the membranes by incubation in stripping buffer (100
26 mM 2-mercaptoethanol, 2 % SDS, and 62.5 mM Tris-HCl;
27 pH=6.7). Incubation was allowed to proceed for 30
28 min. to 1 hour at 50-60°C. The membrane was washed
29 in TBS-T for 1 hour and blocked in 2% casein. At
30 this stage, the membrane was ready for re-incubation
31 with a primary antibody.

32

1 Purification of the recombinant α and β subunits of
2 IL-12

3

4 Ni^{2+} -NTA chromatography

5

6 Purification of hexahistidine-tagged α - and β -chains
7 was performed using nickel-nitrilotriacetic acid
8 (Ni^{2+} -NTA) affinity chromatography. Ni^{2+} -NTA agarose
9 was obtained from QIAGEN.

10

11 Cross-linking of proteins

12

13 Following induction, cells were washed, scraped and
14 resuspended in PBS supplemented with 100 $\mu\text{g}/\text{ml}$ of
15 dithiobis(succinimidylpropionate (DSP). DSP is a
16 homobifunctional NHS-ester that reacts with the ϵ -
17 amines of lysines residues, so as to form a covalent
18 amide bond. Cross-linking reactions were incubated
19 at room temperature for 30 minutes, with
20 intermittent vortexing performed every 5 minutes.
21 Reactions were quenched by adding 100 mM of Tris.HCl
22 ($\text{pH}=8.0$). As Tris contains DSP-reactive primary
23 amines, the aim of this 'quenching' reaction is to
24 block any remaining unreacted DSP. Quenching was
25 allowed to proceed for 15 minutes.

26

27 Inhibitor and cytotoxicity assays

28

29 Inhibitor assay

30

31 To analyse the effect of inhibitors on formation and
32 secretion of IL-12, generally cells were grown in

1 12-well plates. When the cells reached a confluency
2 of 70 %, inhibitors were added to the culture medium
3 at the concentrations indicated. After 2 hours of
4 incubation, cells were induced with ponasterone A.
5 Sixteen to twenty-four hrs later, medium was
6 collected to analyse secretion of α and β -chains,
7 either alone or in combination. Cells were lysed as
8 described above, and soluble and insoluble fractions
9 were prepared. In some experiments, the a- and/or b-
10 chains were purified by means of Ni²⁺-NTA agarose
11 affinity chromatography.

12

13	INHIBITION OF		Concentration
14 A23187	Ionophore		0.1 to 30 μ M
15 CELEBREX	Cox-2 Inhibitor		10 to 100 μ M
16 Thapsigargin	ER Ca-ATPase		5 μ M

17

18

19 Cytotoxicity test

20

21 The mitochondrial ...MTT test is widely use as a
22 cytotoxicity test. This test is principally based on
23 the propensity of mitochondrial dehydrogenases to
24 cleave the tetrazolium ring of. The viability of
25 cells is proportional to the activity of
26 mitochondrial dehydrogenases. Cleavage of the
27 tetrazolium ring results in the formation of purple
28 formazan crystals. We used the MTT assay to quantify
29 cytotoxicity of celecoxib on Ecr293 cells. The test
30 was performed in 96-well plates in which 10⁵ cells
31 per well were plated the day before application of
32 the MTT test. Following addition of celecoxib to the

1 culture medium, cells were induced by ponasterone A,
2 as explained before. After 16 hours of induction,
3 the MTT reagent (10 μ l of 100 mg/ml stock solution)
4 was added to the cells. Two hours later, the medium
5 was removed, and the cells were dissolved in DMSO.
6 DMSO solubilizes formazan crystals. Absorbance was
7 measured at 550 nm using a 96-well plate
8 spectrophotometer.

9

10 Description of the Ecdysone-Inducible Mammalian
11 Expression System

12

13 As a means to study folding and secretion of dimeric
14 forms of interleukin, a series of cell lines that
15 produce the recombinant α and β -chain under
16 transcriptional control of a chemically inducible
17 promotor were developed. The expression system used
18 is based on the ability of the insect hormone
19 ecdysone (analog Ponasterone A) to induce
20 transcription of IL-12 in mammalian cells from a
21 compatible promoter. Since mammalian cells do not
22 express the ecdysone receptor, the basal levels of
23 transcription of IL-12 were low or non-existent. The
24 hormone ecdysone (or its analogs) does not affect
25 the physiology of mammalian cells, and hence, can be
26 used without inducing any other irrelevant or toxic
27 effects. This expression system facilitates
28 extremely tight control of the expression of α and
29 β -chain genes, which is of interest for both kinetic
30 studies and studies in which inhibitors are used as
31 a means to monitor the process of folding and
32 secretion of IL-12.

1
2 Architecture and components of the Ecdysone-
3 Inducible Mammalian Expression System

4
5 The Ecdysone-Inducible Mammalian Expression System
6 (EIMES) is based on the use of a heterodimer
7 composed of the ecdysone receptor (VgEcR) and the
8 retinoid X receptor (RxR) (Figure 1A). Both receptors
9 are coded for in the cell line by the plasmid pVgRxR
10 vector that carries the zeocin resistance gene,
11 allowing for selection by means of this antibiotic.
12 The ecdysone receptor is under transcriptional
13 control of the Rous sarcoma virus promoter (P_{RSV})
14 while the retinoid receptor is located downstream
15 from the cytomegalovirus promoter (P_{CMV}). Both are
16 constitutive promoters facilitating continuous
17 production of high levels of the heterodimer. The
18 ecdysone receptor contains the VP16 transactivation
19 domain which increases the level of induction. In
20 the presence of ponasterone A (ecdysone analog) the
21 ecdysone and retinoid X receptors will bind to each
22 other, and the heterodimerized receptor will
23 subsequently bind to the ecdysone/glucocorticoid
24 response element (E/GRE) sequence present in the
25 promoter of pIND vectors to be used as vehicle for
26 expression of IL-12 chains (Figure 1B). Both
27 receptors have a DNA binding domain (DBD) which
28 recognises half of the response element (E/GRE). The
29 DBD of the ecdysone receptor recognises 5'AGTGCA3'
30 and the DBD of the retinoid receptor recognises the
31 sequence 5' AGAACA3' (Yao et al., 1993). The
32 response element is upstream from the promoter that

1 activates gene expression ($P_{\Delta HSP}$) in pIND. Thus the
2 binding of the receptor heterodimer to these
3 response elements will induce the transcription of
4 the gene of interest (Figure 1B). The cell line used
5 is EcR293, a derivative of the HEK293 cell line that
6 is transfected with the pVgRXR vector and cultivated
7 in the presence of zeocin.

8

9 pIND expression vectors for production of IL-12

10

11 Three different pIND vectors (pIND, pINDSP1 and
12 pINDHygro) are available all of which can be used in
13 this expression system to produce recombinant
14 proteins (Figure 2). All of these contain an
15 ampicillin resistance gene to enable selection and
16 propagation of clones in E. Coli cells. The multiple
17 cloning site is located downstream from a minimal
18 heat shock promoter ($P_{\Delta HSP}$). pIND and pINDSP1 differ
19 from pINDHygro in that the first two vectors contain
20 the neomycin resistance gene while pINDHygro
21 contains the hygromycin resistance gene. These
22 different antibiotic resistance genes allow for dual
23 selection of transfected cells in the presence of
24 both antibiotics. This is important in view of the
25 requirement of producing cell lines that express
26 both subunits of dimeric interleukins, with each
27 subunit provided by a different vector.

28

29 The pINDSP1 vector contains three SP1 binding sites
30 inserted between the response elements and the
31 promoter, which theoretically increases the

1 expression levels five times in comparison with pIND
2 (Kadonaga et al., 1987).

3

4 Rational for use of histidine tags

5

6 The use of the histidine tag as a means for
7 purification of recombinant proteins is a well-
8 documented method proven to be highly efficient.
9 The major advantages of this system are:
10 Purification can be achieved from a mix containing
11 less than 1 % of total protein in one-step.
12 Purification can be completed under native or
13 denaturing conditions since the binding of the
14 histidines to the Ni-NTA agarose is not dependent on
15 the conformation. The His tag is a small tag and it
16 does not interfere with the structure or function of
17 the protein to be expressed so removal of the tag is
18 not necessary. The His tag can be used as the target
19 to be recognized by an antibody anti-His tag. The
20 histidine tag can be engineered so as to be
21 expressed in the target protein in either N-
22 (preceded by ATG initiation codon) or C-terminal
23 (followed by TAA, TGA or TAG stop codon) position.
24 This is accomplished through the use of specific
25 primers which are designed so as to contain the
26 coding sequence for 6 histidines fused to the
27 sequence of our target protein. By means of metal
28 ionic affinity chromatography (matrix used Ni²⁺-
29 nitrilotriacetic acid coupled to agarose,
30 abbreviated as Ni-NTA) His-tagged recombinant
31 proteins can be captured and purified in a highly
32 selective and specific manner. This strategy was

1 applied to the purification of the IL- α and β -
2 chains from both cell lysates (in order to capture
3 protein in the process of folding in the endoplasmic
4 reticulum and to co-capture proteins associated with
5 the folding chains such as chaperones) and medium
6 (so as to capture fully folded and matured secreted
7 protein).

8

9 Amplification of α and β chains of IL-12

10

11 Design of primers

12

13 The composition of the nucleotide sequence preceding
14 the ATG translation initiation codon is known to
15 affect translation initiation. Therefore primers
16 optimized for translation were designed (consensus
17 sequence: GCCGCC ATG). To clone both subunits
18 directionally into the multiple cloning sites of
19 pIND plasmids, an NheI restriction site was
20 introduced in the forward primers and an XhoI
21 restriction site in the reverse primers (Figure 3).
22 The α and β -chain sequences of IL-12 (Sequence ID
23 No.s 6 and 7) (Genbank accession numbers: M65291 and
24 M65290) were checked to assure that none of these
25 contain these restriction sites.

26

27 The IL-12 α -chain sequence contains two initiation
28 codons (ATG), which occur in the same reading frame
29 and are 99 nucleotides apart. It has been
30 demonstrated that α -chains translated from either
31 the first or second start codon are functional.
32 Thus, the initiation codon used may affect the

1 length of the signal peptide, but does not affect
2 primary structure and folding of the mature chain.
3 This is understandable since folding occurs in the
4 ER after the signal peptide has been removed. The
5 forward primer was designed to contain the second
6 start codon of the functional α -chain. The reverse
7 primer contained the stop codon (TAA) and the
8 sequence for six histidines engineered between the
9 carboxy-terminus and the stop codon. Similarly, the
10 β -chain primers contained ATG and TAG stop codons.
11 For the β chain, however, two reverse primers were
12 designed, i.e. one containing the sequence coding
13 for the six histidines and the other without the
14 histidine tag (Figure 3).

15

16 Amplification of the α and β chains of IL-12 by RT-
17 PCR from U937-extracted mRNA

18

19 In order to obtain mRNA of the IL-12 α and β chains,
20 a monocytic cell line (U937) was induced with LPS
21 for 16 hours, a treatment which is known to result
22 in the production of IL-12 in this cell line. The
23 RNA was extracted, and mRNA was retrotranscribed
24 into cDNA by RT-PCR using the primers described in
25 the preceding paragraph and the high-fidelity
26 thermostable Pwo DNA polymerase. Since the
27 concentration of $MgSO_4$ is known to influence the
28 specificity of primer annealing three different
29 concentrations of $MgSO_4$ were used in the PCR
30 reaction. Subsequently, the amplification products
31 were analysed by means of 1.5% agarose gel
32 electrophoresis. Though a band was visible that

1 corresponded to the expected length of the amplified
2 β chain (900 bp; Figure 4), no amplification product
3 was obtained for the α chain (not shown).

4

5 Amplification of the α and β chains of IL-12 by PCR
6 from cDNA

7

8 The α and β -chains were amplified using as template
9 the full-length cDNAs obtained from the ATCC and the
10 HGMP Resource Centre, respectively. Again, we
11 decided to use Pwo DNA polymerase for amplification
12 rather than Taq polymerase, since the former
13 displays 3' \rightarrow 5' exonuclease proof-reading
14 activity which is known to reduce the accumulation
15 of errors in the final PCR product. The reactions
16 were carried out as explained in section 2.1.3. The
17 PCR products obtained by amplification of the cDNAs
18 of the α and β -chains were analyzed by means of 1.5%
19 agarose gel electrophoresis. Figure 5 illustrates
20 the amplification of the α -chain: a PCR product
21 corresponding to 700 bp was specifically amplified
22 in the presence of 2-3 mM MgSO_4 . Figure 6 shows the
23 900-bp PCR product obtained following amplification
24 of the cDNA of the β -chain.

25

26 Construction of pIND-derived expression vectors

27

28 Introduction

29

30 The PCR products were purified and digested with
31 NheI and XhoI, and subsequently cloned into
32 NheI/XhoI-cut vectors. 5 different constructs were

1 created, i.e. pIND-p35H, pIND(SP1)-p35H, pINDHygro-
2 p40, pIND(SP1)-p40H and pIND-p40. The__expression
3 cassettes for the α and β chains of IL-12 contained
4 within these vectors are specified in Figure 7. As
5 explained above, pIND(SP1) and pINDHygro confer
6 resistance to different antibiotics, i.e. neomycin
7 and hygromycin respectively, when expressed in
8 mammalian cells. Thus, expression vectors were
9 constructed that would facilitate selection of the
10 following stable cell lines:

11

- 12 1. EcR293 cells expressing the carboxyterminal-
13 His-tagged α -chain selected by the antibiotic
14 neomycin (transfected with either pIND-p35H or
15 pIND(SP1)-p35H, anticipated to differ only in
16 the level of expression);
- 17 2. EcR293 cells expressing the β -chain selected
18 with neomycin (pIND-p40 or pIND(SP1)-p40H,
19 differing in level of expression but also in
20 the presence or absence of a carboxyterminal
21 His-tag);
- 22 3. EcR293 cells expressing the β -chain selected
23 with hygromycin (pINDHygro-p40)
- 24 4. EcR293 cells expressing the α/β heterodimer
25 selected with both neomycin and hygromycin
26 (pINDHygro-p40 and either pIND-p35H or
27 pIND(SP1)-p35H).

28

29 Selection and sequencing of clones

30

31 Competent E. coli JM109 cells were transformed with
32 these different constructs. Following

1 transformation, the cells were plated on Petri
2 dishes containing LB-agar supplemented with
3 ampicillin. pIND vectors confer resistance to
4 ampicillin to E. coli cells that have successfully
5 integrated the plasmid. However, still the presence
6 or absence of an insert in the vector has to be
7 verified. In order to confirm the presence of the
8 insert three complementary methods were adopted.
9 First, colony PCR was performed facilitating the
10 identification of positive clones by means of direct
11 amplification of the insert using α and β -chain-
12 specific primers. Second, the presence of the insert
13 by NheI/XhoI restriction digestion of plasmid
14 minipreps and electrophoresis. Third, forward and
15 reverse sequencing was performed to validate the
16 presence of the insert and the absence of any
17 errors. The results of the colony PCR procedure are
18 illustrated in Figures 8 and 9, which show that not
19 every ampicillin-resistant colony appeared to
20 contain the insert.

21
22 The positive colonies that were identified in Figure
23 8 and 9 were propagated in LB medium supplemented
24 with ampicillin, and minipreps and glycerol stocks
25 were prepared. To confirm the presence of the
26 insert in the plasmid minipreps were digested with
27 NheI and XhoI restriction enzymes and these products
28 were subjected to 1.5% agarose gel electrophoresis
29 (Figure 10).

30
31 The third method utilised to verify that the
32 plasmids extracted from ampicillin-resistant clones

1 contained the correct inserts corresponding to
2 either α and β -chains, consisted of
3 dideoxynucleotide DNA sequencing. Forward and
4 reverse sequencing was performed using the multiple
5 cloning site primers, i.e. ecdysone forward primer
6 and BGH reverse primer. This showed that error-free
7 inserts were present in the right orientation in
8 each of the vectors.

9

10 Development of stably transfected EcR293 cell lines

11

12 Extraction of endotoxin-free plasmid DNA to be used
13 for transfection of EcR293 cells

14

15 The plasmids were purified using the Endofree
16 purification kit from QIAGEN. This kit facilitates
17 large-scale extraction of plasmid DNA from 100ml of
18 bacterial cultures while efficiently removing
19 endotoxins. Endotoxins are toxic for mammalian
20 cells, and their presence in DNA preparations may
21 decrease transfection efficiency. The DNA of the
22 purified samples was quantified by spectrophotometry
23 (A_{260}). The concentrations obtained ranged between
24 0.4 and 2 $\mu\text{g}/\mu\text{l}$ (Table 1). The purity of DNA samples
25 was calculated by absorption measurements at 260 and
26 280. A ratio $A_{260/280}$ amounting to 1.8 to 2 is
27 indicative for a very high purity. As can be seen in
28 Table 1, both the amounts and purities of the
29 plasmid DNA obtained using the Endofree kit were
30 highly satisfactory.

31

1 Table 1. Concentration, total amount and purity of
2 plasmid DNA extracted from bacterial cultures with
3 the Endofree kit

4

Plasmid	A ₂₆₀	Conc.	Total Amt.	Ratio (Purity)
pIND (SP1) -p35H	0.051	0.577µg /µl	115.4µg	1.825
pIND Hygro -p40	0.070	2.059µg /µl	411.5µg	1.876
pIND -35H	0.097	0.998µg /µl	199.6µg	1.809
pIND -p40	0.047	0.478µg /µl	95.6µg	2.082
pIND (SP1) -p40H	0.098	1.07µg /µl	214µg	1.89

5

6 Transfection and selection of EcR293 cells

7

8 EcR293 cells were transfected with these vectors,
9 either alone or in combinations. Following 1 day of
10 recovery after transfection, cells were trypsinized,
11 diluted and seeded into 96-well plates. The
12 appropriate antibiotics were added to the culture
13 medium to initiate the selection__process. As
14 summarized in Table 2, three different cell
15 concentrations and two different antibiotic
16 concentrations were used to perform selection over
17 time.

1
2 Vectors and vector combinations used to transfect
3 EcR293 cells:

4
5 1-pIND-p35H 3pIND-p40 6-pIND-p35H/pINDHygro-40
6 2-pIND- 4pINDHygro 7-pIND(SP1)-35H/pINDHygro
7 (SP1)-p35H -p40 -p40
8 5pIND(SP1)
9 -p40H

10
11 Table 2. Cell and antibiotic concentrations for
12 selection of transfected EcR293 cells

	Conc. Neomycin	Conc. Hygromycin	Conc. Zeocin
10 ⁶ transfected cells	Dilution 1/10 (10 ⁵ cells/well)	300 µg/ml	300 µg/ml
		600 µg/ml	400 µg/ml
	Dilution 1/100 (10 ⁴ cells/well)	300 µg/ml	300 µg/ml
		600 µg/ml	400 µg/ml
	Dilution 1/1000 (10 ³ cells/well)	300 µg/ml	300 µg/ml
		600 µg/ml	400 µg/ml

13 For the construct made with the pINDHygro vector
14 (pINDHygro-p40), selection was performed in the
15 presence of either 300 or 600 µg/ml hygromycin.
16 These concentrations were chosen on the basis of the
17 concentrations of hygromycin recommended by the
18 manufacturer of the pIND series of vectors for
19 selection of transfected EcR293 cells (between 200
20 and 600µg/ml). Similarly, cells transfected with
21 pIND- and pINDSP1-derived vectors were cultivated in
22 the presence of either 300 or 600 µg/ml neomycin, as
23 recommended. Hygromycin concentration of 200 µg/ml

1 was used in all further transfection experiments
2 with pINDHygro-p40. After 6 weeks we were able to
3 detect about 40 different clones in total, generated
4 by transfection with the different constructs and
5 selection with the appropriate antibiotics.

6

7 Immunodetection of expression of α and β chains
8 following induction with ponasterone A

9

10 As a test in order to evaluate whether these clones
11 were able to produce the corresponding recombinant
12 proteins, we selected three clones, i.e. 1 single
13 clone for pIND-p35H (clone 1A9), 1 for pIND(SP1)-
14 p35H (clone 2G10) and 1 for pIND-p40 (clone 3D9).
15 These clones were trypsinized and plated into the
16 wells of 6-well plates. The cells were induced with
17 Ponasterone A (5 μ M) for 48 hours. Subsequently, the
18 cell culture medium was collected, and the cells
19 were lysed. This was done to evaluate the presence
20 of the recombinant protein in both secreted and
21 intracellular fractions. Culture medium and soluble
22 cytoplasmic fractions were subjected to 4-15%
23 reducing SDS-PAGE (Figure 11). The proteins were
24 transferred by electroblot to a PVDF membrane.
25 Immunodetection was performed with anti-IL-12 α - or
26 β -chain antibodies. Immunoreactive bands were
27 visualized using a chemoluminescence-based kit and
28 autoradiography films, Kodak BioMax MR films. (ECL
29 kit; see sections 2.7).

30

31 This first analysis indicated that p40 is more
32 efficiently secreted than p35, as the ratio of

1 secreted/intracellular is obviously higher for the
2 former. Finally, a band corresponding to the Mr of
3 serum albumin was visible in all immunoblots of
4 medium fractions (indicated with arrow in Figure 11
5 A and B). A similar immunoreactive band was found in
6 the medium of uninduced or untransfected cells,
7 indicating that this band is unrelated to any of the
8 IL-12 chains but is likely visualized following a-
9 specific interaction with either the primary or
10 secondary antibodies used in these experiments (not
11 shown).

12

13 Differences in expression levels in stably
14 transfected cell lines

15

16 Having demonstrated the inducible expression of
17 immunoreactive proteins corresponding to either the
18 α or the β chain of IL-12 in some of the Ecr293 cell
19 clones produced, the expression levels in all of the
20 clones were evaluated by means of a similar
21 procedure. For this purpose cells, precedingly
22 seeded in 96 well plates (5×10^4 cells) were induced
23 with ponasterone A for 24 hours. Induced and
24 uninduced cells were lysed in 6 μ l of lysis buffer,
25 and the lysates were subjected to 4-15% reducing
26 SDS-PAGE and immunoblot (Figure 12 and 13).

27

28 Surprisingly, an anti- α -chain reactive band was
29 observed in the lysates of both un-induced and
30 induced Ecr293 cells that exhibited a slightly lower
31 Mr than the inducible, recombinant α -chain. This
32 band was also consistently observed in immunoblots

1 of un-transfected EcR293 cells (not shown). Thus,
2 this protein is likely to correspond to a natural,
3 constitutively produced form of either p35 or a p35-
4 related protein in these cells. Its Mr is smaller
5 than that of the recombinant form, which is likely
6 due to the absence of the hexahistidine-tag in the
7 natural form. Nevertheless, the smaller form is
8 unlikely to correspond to a proteolytically
9 generated truncated form of the recombinant his-
10 tagged α -chain as it is equally present in un-
11 induced or un-transfected cells.

12

13 Most of the cell lines were freezed and kept in
14 liquid nitrogen. Cell line 2B9 (Figure 12, lane 1-
15 2), which appeared to be the cell line with the
16 highest expression level of the α -chain was
17 maintained in cultivation for further experiments.
18 This cell line was re-named HACHIE.1. Similarly,
19 cell line 3H10 which expresses high levels of the β -
20 chain (Figure 13B, lane 1-2) was maintained in
21 culture. This cell line was re-named HIBERNIA.1.

22

23 Transient transfection of HIBERNIA.1 cells to
24 produce heterodimeric IL-12

25

26 As described above, HIBERNIA.1 is a cell line that
27 produces high levels of carboxyterminally
28 hexahistidine-tagged β -chain upon induction with
29 ponasterone A, and was obtained by transfection of
30 EcR293 cells with pIND(SP1)-p40H followed by
31 selection with neomycin. The transient transfection
32 was carried out in 6-well plates using 1 or 2 μ g of

1 endotoxin-free pIND(SP1)-p35H plasmid DNA. Cell
2 culture medium was collected at 30 and 48 hours
3 following induction. The samples were run in a non-
4 reducing gel so as to facilitate detection of the
5 disulfide-bonded heterodimer. Following
6 electrophoresis, semi-dry blotting was performed,
7 and the membrane was successively probed with an
8 anti- β -chain (Figure 14) and an anti- α -chain
9 antibody (Figure 14).

10

11 Figure 14 shows that in the culture medium of both
12 the transiently transfected (lanes 1 to 4) and not-
13 transfected (lane 5) HIBERNIA.1 cells 2 immuno-
14 reactive bands are detected with the anti- β -chain
15 antibody, with Mr's of about 40 and 80 kD
16 respectively. In lanes 1 to 4, the 80-kD band could
17 represent the β chain homodimer (2x40 kD) as well
18 as the α/β chain heterodimer (35+40 kD), as both
19 would migrate as bands with similar Mr in this low-
20 resolution SDS-PA gel. In not-transfected HIBERNIA.1
21 cells (lane 5 of Figure 14) the 80 kD band must
22 necessarily represent the β chain homodimer. Figure
23 14 shows that a 80-kD protein band which is reactive
24 with the anti- α -chain antibody is present only in
25 HIBERNIA.1 cells transfected with pIND(SP1)-p35H
26 (lanes 1 to 4) but not in un-transfected HIBERNIA.1
27 cells (lane 5). Analysis of recombinant cell lines
28 secreting the α chain by means of non-reducing SDS-
29 PAGE showed that the α chain is present only as a
30 monomer form when expressed in the absence of the β
31 chain (data not shown). In view of these findings,
32 it can be safely concluded that HIBERNIA.1 cells

1 transiently transfected with pIND(SP1)-p35H secrete
2 the α/β disulfide-bonded IL-12 heterodimer upon
3 induction with ponasterone A. In fact, in these
4 cells the total amount of α chain secreted ends up
5 as subunit of the heterodimer form, as anti- α -chain
6 reactivity is only visible as an 80-kD band and not
7 as a 35-kD band. However, it is likely that a
8 certain fraction of the β chain produced in
9 transiently transfected HIBERNIA.1 cells will still
10 be present as homodimer. This possibility is
11 difficult to exclude in view of the fact that the
12 non-transfected HIBERNIA.1 cells produce the β
13 homodimer.

14

15 Transfection of HIBERNIA.1 cells with with 1 μ g
16 pIND(SP1)-p35H resulted in a higher
17 production/secretion of the heterodimer compared to
18 transfection with 2 μ g. This might be related to the
19 fact that due to the 1:1 stoichiometry of α and β
20 chain interaction in the heterodimer, a level of α -
21 chain production which is higher than that of the β
22 chain may be counterproductive for efficient
23 formation of the heterodimer.

24

25 To verify the composition of the 80-kD band secreted
26 by transiently transfected HIBERNIA.1 cells, we run
27 the medium collected at 48 hrs after induction from
28 HIBERNIA.1 cells transfected with 1 μ g of pIND(SP1)-
29 p35H (* in Figure 14), again, this time in a
30 reducing gel. Gels were blotted, and detection was
31 carried out with either the anti- α -chain antibody,

1 the anti- β -chain antibody or with both antibodies at
2 the same time.

3

4 The anti- α -chain antibody detected a band
5 corresponding to 35 kD, while the anti- β -chain
6 antibody detected a band of approximately 40 kD
7 (Figure 15). Thus, the Mr's of the α and β chains
8 produced in transiently transfected HIBERNIA.1 cells
9 coincide with those theoretically predicted. The α
10 chain appeared as a more diffuse band than the β
11 chain. This is most likely due to more extensive
12 heterogeneity in N-glycosylation of the former, as
13 tunicamycin treatment produced a much sharper α -
14 chain band (demonstrated below).

15

16 This data shows that a genuinely processed α -chain
17 form is produced in transiently transfected
18 HIBERNIA.1 cells that interacts with the β -chain to
19 form a disulfide-linked secreted IL-12 heterodimer.
20 Obviously, these experiments show that attachment of
21 hexahistidine-tags to the carboxytermini of both the
22 α - and β -chains does not interfere with correct
23 folding, assembly and secretion of the heterodimer.

24

25 Capture of α/β - and β/β IL-12-H6-chaperone complexes
26 on Ni^{2+} -NTA

27

28 Following induction with Ponasterone A, cells were
29 lysed. - α/β and β/β -H₆-chaperone complexes were
30 captured on Ni^{2+} -NTA agarose. The gel was washed 5
31 times with buffer A (100mM NaH_2PO_4 , 10mM TrisHCl, 8M
32 urea, pH 6.3), and elution was carried out with

1 buffer B (same as Buffer A, but pH 4.3). Complexes
2 were boiled in SDS loading solution + DTT. Proteins
3 were separated by 4-15% SDS-PAGE and transferred to
4 PVDF membranes. Detection was carried out using
5 anti-p35 antibody G161-566.14 (Pharmingen).
6 Membranes were stripped and re-probed successively
7 with anti-chaperone antibodies (α -CRT, α -Grp78, α -
8 -Grp94 & α -CNX; StressGen).
9

10 Experimental findings

11

12 IL-12 is a secretory protein. Secretory proteins are
13 defined as proteins that are released by cells into
14 the extracellular milieu, and that exert their
15 biological activity by binding onto a specific
16 membrane receptor of target cells. 'Folding' (i.e.
17 generation of a correct three-dimensional structure)
18 of secretory proteins, such as IL-12, typically
19 occurs in a membrane-surrounded cell organelle,
20 named the endoplasmic reticulum (ER). The ER is
21 specifically enriched in chaperones, thioredoxin-
22 type isomerases and proteins involved in
23 glycosylation pathways. An important role of these
24 factors is to assist in ensuring correct folding of
25 secretory proteins during their transit in the ER
26 prior to their secretion into the extracellular
27 milieu. Improperly folded secretory proteins are
28 generally retained in the ER and subsequently
29 degraded by proteases and components of the
30 cytosolic proteasome. It was hypothesised that the
31 use of selected pharmacological agents that
32 interfere with the proper functioning of 'folding'-

1 assisting factors in the ER could be used to inhibit
2 proper folding, and, hence, secretion of IL-12.
3 As a first step, different tightly controlled
4 ecdysone-inducible recombinant cell lines expressing
5 functional C-terminally hexahistidine-tagged IL-12
6 α/β (heterodimer) and IL-12 β/β (homodimer) chains
7 were developed. The use of such recombinant cell
8 lines alleviates some of the problems related to the
9 use of natural producer cells of IL-12 (e.g.
10 restricted availability, lack of reproducibility
11 etc). These recombinant cell lines were used as a
12 means to study the processes that determine
13 regulation of folding, assembly and secretion of IL-
14 12 homo- and heterodimers. The following inhibitors
15 were used: (i) thapsigargin (an ER Ca^{2+} -ATPase
16 inhibitor), and (ii) the ionophore A23187 and (iii)
17 celecoxib (a putative ER Ca^{2+} perturbing reagent),
18 each over a wide range of concentrations.
19
20 Following a 16-hr treatment of cells with these
21 inhibitors, culture medium was collected and the
22 presence of secreted IL-12 forms was detected by
23 means of non-reducing SDS-PAGE and western
24 immunoblot. It was found that neither the α/β nor
25 the β/β dimer forms of IL-12 were present in the
26 culture medium of cells treated with thapsigargin
27 when this was added over a concentration range of
28 0.1 μM to 15 μM . The amount of extracellularly
29 secreted IL-12 dimer forms produced by thapsigargin-
30 treated cells was <5% of that produced by untreated
31 cells (maximal suppression was observed for all
32 concentrations of thapsigargin greater than or equal

1 to 0.1 μM). Similarly, the calcium ionophore A23187
2 suppressed formation of secreted IL-12 dimer forms
3 when it was used over a concentration range of 0.1
4 μM to 30 μM , with maximal suppression (>95% compared
5 to untreated cells) from 1 μM . Toxicity conferred by
6 these inhibitors over the test period of 16 hr as
7 measured with the MTT test was observed for
8 concentrations of thapsigargin >5-10 μM and for
9 concentrations of A23187 >10 μM . Thus, the maximal
10 suppression of secreted IL-12 dimer production is
11 achieved at an inhibitor concentration at which
12 toxic effects are totally absent, showing that both
13 IL-12-suppressive and cell-toxic effects conferred
14 by these inhibitors are independent. Secretion of
15 IL-12 α and β monomer forms was suppressed by
16 neither thapsigargin nor A23187.

17
18 Both thapsigargin and A23187 are likely to exert
19 these effects by decreasing the concentration of Ca^{2+}
20 in the ER. It is likely that the resulting
21 suboptimal concentration of Ca^{2+} in the ER blocks the
22 activity of Ca^{2+} -dependent chaperones and folding-
23 assisting proteins involved in the dimer formation
24 of IL-12. It was investigated whether CELECOXIB can
25 be used to suppress production of secreted IL-12
26 dimer forms.

27
28 Celecoxib was dissolved in DMSO and added to
29 recombinant HEK293 cells over a concentration range
30 from 10 μM to 100 μM . As a control DMSO-only treated
31 cells were used. Celecoxib concentrations were
32 chosen on the basis of available literature data,

1 and coincide with optimal activity of the compound
2 in various cell-based systems. Two hours later cells
3 were induced with Ponasterone A to produce IL-12 α/β
4 or β/β dimer forms. After 16 hrs of additional
5 incubation, culture medium was collected and
6 assessed for the presence of IL-12 dimer forms by
7 means of non-reducing SDS-PAGE and immunoblot. This
8 showed that Celecoxib suppressed production of
9 secreted IL-12 β/β homodimers by >95% when used at a
10 concentration equal to or larger than 30 μM ; and of
11 secreted IL-12 α/β heterodimers by >95% when used
12 at a concentration equal to or larger than 10 μM .
13 Secretion of IL-12 α and β monomer forms was not
14 suppressed by Celecoxib. Toxicity as measured with
15 the MTT assay was visible when cells were treated
16 for 16 hrs with a concentration of Celecoxib equal
17 to or larger than 100 μM .

18

19 The present data demonstrates that Celecoxib
20 efficiently suppresses secretion of IL-12 α/β and
21 β/β dimer forms by a post-transcriptional and post-
22 translational mechanism that involves Ca^{2+} -dependent
23 intracellular retention of IL-12 dimers. Maximal IL-
24 12-suppressive effects are observed at a
25 physiological Celecoxib concentration in the absence
26 of any obvious toxic effects.

27

28 For oral administration, the medicament according to
29 the invention may be in the form of, for example, a
30 tablet, capsule suspension or liquid. The medicament
31 is preferably made in the form of a dosage unit
32 containing a particular amount of the active

1 ingredient. Examples of such dosage units are
2 capsules, tablets, powders, granules or a
3 suspension, with conventional additives such as
4 lactose, mannitol, corn starch or potatoes starch;
5 with binders such as crystalline cellulose,
6 cellulose derivatives, acacia, corn starch or
7 gelatins; with disintegrators such as corn starch,
8 potato starch or sodium carboxymethyl-cellulose;
9 and with lubricants such as talc or magnesium
10 stearate. The active ingredient may also be
11 administered by injection as a composition wherein,
12 for example, saline, dextrose or water may be used
13 as a suitable carrier.

14
15 For intravenous, intramuscular, subcutaneous, or
16 intraperitoneal administration, the compound may be
17 combined with a sterile aqueous solution which is
18 preferably isotonic with the blood of the recipient.
19 Such formulations may be prepared by dissolving
20 solid active ingredient in water containing
21 physiologically compatible substances such as sodium
22 chloride, glycine, and the like, and having a
23 buffered pH compatible with physiological conditions
24 to produce an aqueous solution, and rendering said
25 solution sterile. The formulations may be present in
26 unit or multi-dose containers such as sealed
27 ampoules or vials.

28
29 If the inflammatory disease is localized in the G.I.
30 tract, the compound may be formulated with acid-
31 stable, base-labile coatings known in the art which
32 began to dissolve in the high pH intestine.

1 Formulations to enhance local pharmacologic effects
2 and reduce systemic uptake are preferred.

3

4 Formulations suitable for administration
5 conveniently comprise a sterile aqueous preparation
6 of the active compound which is preferably made
7 isotonic. Preparations for injections may also be
8 formulated by suspending or emulsifying the
9 compounds in non-aqueous solvent, such as vegetable
10 oil, synthetic aliphatic acid glycerides, esters of
11 higher aliphatic acids or propylene glycol.

12

13 Formulations for topical use include known gels,
14 creams, oils, and the like. For aerosol delivery,
15 the compounds may be formulated with known aerosol
16 excipients, such as saline and administered using
17 commercially available nebulizers. Formulation in a
18 fatty acid source may be used to enhance
19 biocompatibility. Aerosol delivery is the preferred
20 method of delivery for epithelial airway
21 inflammation.

22

23 For rectal administration, the active ingredient may
24 be formulated into suppositories using bases which
25 are solid at room temperature and melt and dissolve
26 at body temperature. Commonly used bases include
27 cocoa butter, glycerinated gelatin, hydrogenated
28 vegetable oil, polyethylene glycols of various
29 molecular weights, and fatty esters of polyethylene
30 stearate.

31

1 The dosage form and amount can be readily
2 established by reference to known inflammatory
3 disease treatment or prophylactic regimens. The
4 amount of therapeutically active compound that is
5 administered and the dosage regimen for treating a
6 disease condition with the compounds and /or
7 compositions of this invention depends on a variety
8 of factors, including the age, weight, sex and
9 medical condition of the subject, the severity of
10 the disease, the route and frequency of
11 administration, and the particular compound
12 employed, the location of the inflammatory disease,
13 as well as the pharmacokinetic properties of the
14 individual treated, and thus may vary widely. The
15 dosage will generally be lower if the compounds are
16 administered locally rather than systemically, and
17 for prevention rather than for treatment. Such
18 treatments may be administered as often as necessary
19 and for the period of time judged necessary by the
20 treating physician. One of skill in the art will
21 appreciate that the dosage regime or therapeutically
22 effective amount of the inhibitor to be
23 administered may need to be optimized for each
24 individual. The pharmaceutical compositions may
25 contain active ingredient in the range of about 0.1
26 to 2000mg, preferably in the range of about 0.5 to
27 500mg and most preferably between about 1 and 200
28 mg. A daily dose of about 0.01 to 100mg/kg body
29 weight, preferably between about 0.1 and about
30 50mg/kg body weight, may be appropriate. The daily
31 dose can be administered in one to four doses per
32 day.

1
2 Although the data presented is based predominantly
3 on the provision of cell lines that when induced
4 produce either homodimeric or heterodimeric IL-12,
5 or either subunit of IL-12, the invention is also
6 applicable in the production of cell lines which
7 when induced produce either IL-23 and IL-27, or
8 subunits thereof. In the case of IL-23, a suitable
9 host cell, such as one which includes an ecdysone-
10 inducible mammalian expression system as described
11 herein, is transformed with a first expression
12 vector according to the invention which includes DNA
13 coding for the p40 (beta) subunit of IL-12 (which is
14 identical to the p40 subunit of IL-23) and a second
15 expression vector which includes DNA coding for the
16 p19 subunit of IL-23. In this regard, the cDNA
17 sequence of the p19 subunit of IL-23 is provided in
18 Sequence ID No. 8. The cDNA is processed by the same
19 restriction enzymes as used with the respective
20 subunits of IL-12, and is ligated into, for example,
21 a pIND vector in the same manner as is described
22 above. Likewise, expression vectors having DNA
23 coding for one of the subunits of IL-27, and cell
24 lines transfected with such expression vectors, may
25 be produced using the techniques described herein.
26
27 The invention is not limited to the embodiments
28 hereinbefore described which may be varied in detail
29 without departing from the invention.